

Raxofelast, a hydrophilic vitamin E-like antioxidant, stimulates wound healing in genetically diabetic mice

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Background. Impaired wound healing is a well-documented phenomenon in experimental and clinical diabetes. Emerging evidence favors the involvement of free radicals in the pathogenesis of diabetes-related healing deficit. This study assessed the effect of systemic administration of raxofelast, a protective membrane antioxidant agent, on wound healing by using healing-impaired (db/db) mice.

Methods. The wound healing effect of raxofelast was investigated by using an incisional skin-wound model produced on the back of female diabetic C57BL/KsJ db+/db+ mice and their healthy littermates (db+/+m). Animals were then randomized to the following treatment: raxofelast (15 mg/kg/d intraperitoneally) or its vehicle (dimethyl sulfoxide/sodium chloride 0.9%, 1:1, vol/vol). The animals were killed on different days, and the wounded skin tissues were used for histologic evaluation and for analysis of malondialdehyde (MDA) level and myeloperoxidase (MPO) activity, wound breaking strength, and collagen content.

Results. Diabetic mice showed delayed wound healing together with low collagen content, breaking strength, and increased MDA levels and MPO activity when compared with their healthy littermates. The administration of raxofelast did not modify the process of wound repair in healthy (db/+) mice, but significantly improved impaired wound healing in diabetic mice through the stimulation of angiogenesis, reepithelialization, synthesis, and maturation of extracellular matrix. Furthermore, raxofelast treatment significantly reduced MDA levels, MPO activity, and increased the breaking strength and collagen content of the wound.

Conclusions. The current study provides evidence that raxofelast restores wound healing to nearly normal levels in experimental diabetes-impaired wounds and suggests that an increased lipid peroxidation in diabetic mice may have a role in determining a defect of wound repair. (*Surgery* 2001;129:467-77.)

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WOUND HEALING IS a complex programmed sequence of cellular and molecular processes, including inflammation, cell migration, angiogenesis, provisional matrix synthesis, collagen deposition, and reepithelialization.¹ The healing process

requires a sophisticated interaction among inflammatory cells, biochemical mediators, extracellular matrix molecules, and microenvironmental cell population.

Impaired wound healing may be a consequence of normal aging, metabolic derangements, or therapeutic intervention.

Raxofelast (IRFI 016; 2,3-dihydro-5-hydroxy-4,6,7-trimethyl-2-benzofuranacetic acid) is a new synthetic analogue of vitamin E with a hydrophilic character and powerful antioxidant properties.²⁻⁴ This compound is quantitatively bioconverted in vivo to the deacetylated active metabolite IRFI 005, which has been shown to effectively scavenge reactive oxygen species and to be a potent inhibitor of lipid peroxidation.^{5,6}

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Table I. Histologic parameters

| | |
|---------------------|--|
| Reepithelialization | Epidermal elongation, differentiation and keratinization; spongiosis; presence of scab; epithelial glycogen accumulation to the margins of the wound; dermo-epidermal junction and epithelial basal membrane |
| Granulation tissue | Thickness of the granulation tissue; collagen matrix formation and organization; degree of edema; fibroblasts number, organization and morphologic features |
| Angiogenesis | New capillary number and position (in the margin of the wound area; in the entire wound area; disposed vertically toward the epithelial wound area) |
| Infiltrated cells | Polymorphonuclear cells, lymphocytes, macrophages: number; scattered in the wound area; only in perivascular or intravascular site |

The effects of raxofelast on the healing process were investigated by using an incisional skin wound model in genetically diabetic mice (db/db mice). Db/db mice are useful as an animal model for wound healing studies because wound healing in these animals is markedly delayed when compared with nondiabetic littermates.^{7,8} Healing impairment is characterized by delayed cellular infiltration and granulation tissue formation, reduced angiogenesis, and decreased collagen and its organization.⁹⁻¹² The mechanism of this alteration is thought to be the result of diabetes production of reactive free radicals that cause lipid peroxidation and impair endothelial cells, fibroblasts and collagen metabolism.¹³ The potential sources of free radical generation in diabetes are ischemia, hyperglycemia by auto-oxidative peroxidation, and leukocytes.

We report that systemic administration of raxofelast reverses the wound healing deficit in diabetic mice by inhibiting lipid peroxidation.

MATERIALS AND METHODS

Animals and experimental protocol. All animal procedures were in accordance with the declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals.

Genetically diabetic female C57BL/KsJ db+/db+ mice and their healthy (db+/+m) littermates, both aged 10 weeks, were obtained from Jackson Laboratory (Bar Harbor, Me).

During the experiments, the animals were housed 1 per cage, maintained under controlled environmental conditions (12-hour light/dark cycle, temperature approximately 23°C), and provided with standard laboratory food and water ad libitum.

The animals were divided into 4 groups (21 animals each). The first and second groups, consisting respectively of diabetic and healthy mice, were given raxofelast at a dose of 15 mg/kg intraperitoneally for 12 days. The third group of diabetic mice and the fourth group of healthy mice were

treated with vehicle (dimethylsulfoxide/sodium chloride 0.9%, 1:1, vol/vol for 12 days).

After general anesthesia was administered with ketamine hydrochloride (110 mg/kg), the hair on the back was shaved and the skin washed with povidone-iodine solution and wiped with sterile water. Two full-thickness longitudinal incisions (4 cm) were made on the dorsum of the mice, and the wound edges were closed with skin clips placed at 1-cm intervals. Seven animals from each group were killed after 3, 6, and 12 days, respectively; and the wounds were divided in 3 segments (0.8 cm wide). The caudal and cranial strip was used for histology while the central one was used for biochemical analysis and wound-breaking strength measurements (on day 12).

Histologic evaluation. The samples were fixed in 10% buffered formalin for light microscopic examination. After fixing, perpendicular sections to the anterior-posterior axis of the wound were dehydrated with graded ethanols and embedded in paraffin. Sections, 5µ thick, of paraffin-embedded tissues were stained with hematoxylin and eosin, periodic acid-Schiff (PAS), and Weigert-van Gieson stains. As part of the histologic evaluation, 6 slides of each biopsy (in particular, 2 hematoxylin-and-eosin-stained sections, 2 periodic acid-Schiff stained sections, and 2 Weigert-van Gieson-stained sections) were examined by 2 independent pathologists without knowledge of the previous treatment, who used masked slides under the microscope from × 20 to × 100 magnification. The parameters measured (Table I) were: reepithelialization, granulation tissue formation, angiogenesis, infiltrated inflammatory cells. The margins of the wound in each of the sections and normal control wounds were used as comparison for scoring (Table II, Fig 1). The histologic score used in this study was evaluated according to literature data regarding wound healing in experimental models.^{5,14}

Breaking strength. The maximum load (breaking strength) tolerated by wounds was measured in a blinded manner on coded samples by using a cal-

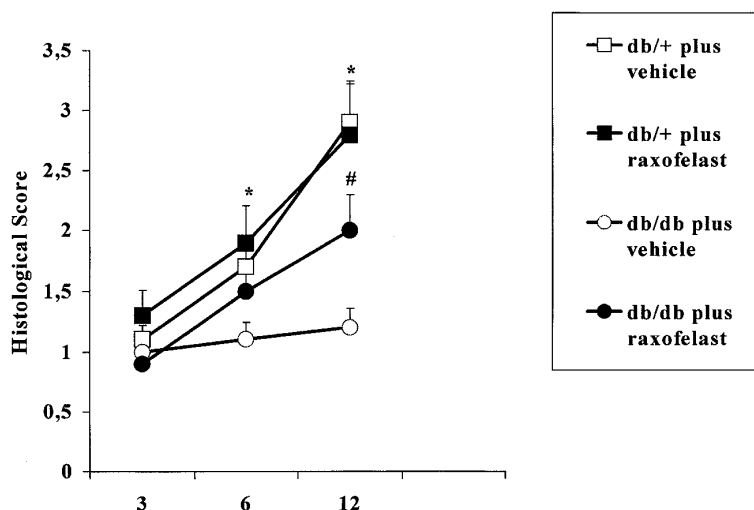


Fig 1. Time course of histological score in db/+ and db/db mice treated with vehicle (1 mL/kg/d, intraperitoneally) and raxofelast (15 mg/kg/d, intraperitoneally). Each point represents the mean \pm SEM of 7 experiments. Asterisk, $P < .01$ versus db/+ mice; number sign, $P < .01$ versus db/db mice treated with vehicle.

ibrated tensometer (Instron Corp, Canton, Mass) as previously described.¹⁵ The ends of the skin strip were pulled at a constant speed (20 cm/min), and breaking strength was expressed as the mean maximum level of tensile strength (g/mm) before separation of wounds.

Biochemical analysis

Malondialdehyde measurement. An assay of lipid peroxidation in wounded skin tissue was done by measuring the level of malondialdehyde (MDA), which is the end product of lipid peroxidation. After being taken, tissue samples were frozen immediately in liquid nitrogen and then stored at -70°C until the assay. The day of analysis, after the thaw, tissue samples were washed in ice-cold 20 mmol/L TRIS-hydrogen chloride (HCL), pH 7.4, blotted on absorbent paper, and weighed. Each sample was then minced in ice-cold 20 mmol/L TRIS-HCL pH 7.4 and homogenized in a 1:10 ratio, weight/volume, by using a nonstick pestle. After centrifugation at 3000g for 10 min at 4°C , the clear homogenate supernatant was used for biochemical assay. The assay was carried out by using a colorimetric commercial kit (Lipid peroxidation assay kit, cat. No. 437634; Calbiochem-Novabiochem Corp).

Briefly, 0.65 mL 10.3 mmol/L N-methyl-2-phenylindole in acetonitrile were added to 0.2 mL homogenate supernatant. After vortexing for 3 to 4 seconds and adding 0.15 mL of HCL 37%, samples were mixed well and closed with a tight stopper and incubated at 45°C for 60 minutes. The samples were then cooled on ice, and the absorbance was measured spectrophotometrically at 586 nm. A cal-

Table II. Scoring of histologic parameters

| Score | Features |
|---------|--|
| 1 \pm | Little epidermal and dermal organization, few capillary vessels, many infiltrated cells |
| 2 \pm | Moderate epidermal and dermal organization, newly formed capillary vessels in the entire wound area, few inflammatory cells |
| 3 \pm | Complete remodeling of the epidermis and dermis, well-formed capillary vessels, few inflammatory cells in perivascular or intravascular site |

ibration curve of an accurately prepared standard malondialdehyde solution (from 2 to 128 nmol/mL) was also run for quantitation.

Myeloperoxidase activity. Myeloperoxidase (MPO) activity, an index of polymorphonuclear leukocyte accumulation, was determined as previously described by Mullane et al.¹⁶ The samples were first homogenized in a solution containing 20 mmol/L potassium phosphate buffer (pH 7.4), 0.01 mol/L EDTA, 50 U/mL of a protease inhibitor (aprotinin) in proportions of 1:10 (wt/vol) and then centrifuged for 30 minutes at 20,000g at 4°C . The supernatant of each sample was then discarded, and the pellet was immediately frozen on dry ice. The samples were kept at a temperature of 0°C for 14 hours before sonication. After thawing, the resulting pellet was added to a buffer solution consisting of 0.5% hexa-decyl-trimethyl-ammonium bromide (Sigma, St Louis, Mo) dissolved in 50 mmol/L potassium phosphate buffer (pH 6) con-

Table III. Main morphologic characteristics of the wound

| Mice | Treated db/db mice | Nontreated db/db mice | Nontreated db/+ mice, Treated db/+ mice |
|--------------------|--|---|---|
| Angiogenesis | Well-oriented and well-formed vessels in the edge and in the central site of the wound | Small vessels scattered in the entire wound area | Vessels disposed vertically toward the epithelial surface in the edge site of the wound |
| Fibroblasts | Oriented parallel to the surface of the wound | Scattered throughout the granulation tissue | Spindle fibroblasts well-oriented to the surface of the wound |
| Inflammatory cells | Scattered in the dermis and in perivascular site | Scattered in the dermis | Mainly in the vessels lumina |
| Epithelium | Almost completely remodeled | Disarranged | Complete remodeling |
| Dermis | Slight edema, well-formed collagen | Few collagen fibrils or collagen bundles, moderate edema. | Complete remodeling |

taining 30 U/mL of a protease inhibitor. Each sample was then sonicated for 1 minute at intensity 2 and at a temperature of 4°C. After sonication, the samples were chilled on ice for approximately 30 minutes and centrifuged for 30 minutes at 40,000g at 4°C. An aliquot of the supernatant was then allowed to react with 0.167 mg/mL o-dianisidine dihydrochloride (Sigma) and 0.0010% hydrogen peroxide, and the rate of change in absorbance was measured at 405 nm in a microtiter plate reader. *MPO activity* was defined as the quantity of enzyme degrading 1 μ mol of peroxide/min at 25°C and was expressed in milliunits per gram of weight (mU/g tissue).

Hydroxyproline analysis. Because hydroxyproline (HP) is found almost exclusively in collagen, it is possible to use HP content as an indicator of the amount of collagen present in tissues. Wound HP concentration was determined as described previously.¹⁷ Briefly, frozen wound tissue was hydrolyzed in 2.0 mL 6 N HCl for 3 hours at 130°C. The solution was neutralized to pH 7.0 with 2.5 N sodium hydroxide and diluted 40-fold with deionized water. Two milliliters of diluted solution were mixed with 1.0 mL 0.05 mol/L chloramine-T solution and incubated for 20 minutes at room temperature. One milliliter of 20% p-dimethylaminobenzaldehyde was then added, and the solution was incubated for 20 minutes at 60°C. The absorbance of each sample was determined at 557 nm, and the amount of HP was determined by comparison to a standard curve.

Drug. Raxofelast was generously supplied by Biomedica Foscama Research Centre, Ferentino, (FR), Italy. The compound was administered intraperitoneally in dimethyl sulfoxide/sodium chloride 0.9%, (1:1; vol/vol). All substances were

prepared fresh daily and administered in a volume of 1 mL/kg.

Statistical analysis. All data were analyzed by the Student paired *t* test. The results were expressed as means \pm SEM. The level for statistical significance was set at $P < .05$.

RESULTS

Histologic results. Fig 1 shows the histologic score of wounds throughout the experiment. Db/+ mice had a progressive increase in histologic score culminating at day 12 in complete wound closure (histologic score, 2.9 ± 0.4). In contrast, db/db mice had an impaired wound healing characterized by a lower histologic score (1.09 ± 0.1 at day 12) indicating a markedly delayed wound healing process (Fig 1). Treatment with raxofelast significantly improved the histologic score in db/db mice (2.1 ± 0.25). Furthermore, raxofelast administration did not modify wound repair in db/+ mice.

Qualitative data regarding histologic evaluation at day 12 are summarized in Table III. A large number of neutrophils were mixed with fibroblasts in an edematous tissue, and little dermal and epidermal organization was observed in diabetic mice treated with vehicle. The epithelium showed little epidermal elongation under the scab, spongiosis, intraepithelial bullae containing red blood cells, or debris (Fig 2). Several stellate and spindle fibroblasts were scattered in the granulation tissue. The wound area was characterized by few collagen fibrils and collagen bundles and by a medium to high degree of edema. Few capillary vessels were present in the wound area. The new vessels showed little basement membrane, thus the granulation tissue was edematous and thicker, but less well arranged, than in diabetic mice treated with raxofelast.

In diabetic mice treated with raxofelast, reepithelialization was moderate to complete, showing normal differentiation and keratinization with epidermal elongation spreading over two thirds of the wound surface (Fig 3) and moderate glycogen storage to the margins of the wound area. The granulation tissue was apparently well-organized. Dermal regeneration was characterized by granulation tissue rich in fibroblasts generally oriented parallel to the epidermal layer, moderate collagen fibrils, and collagen bundles, and slight edema (Fig 4). Newly formed capillary vessels were observed in moderate numbers in the dermis of the entire wound area. Vessels were similar to those found in normal dermis. The degree of infiltrated inflammatory cells was minimal, with few polymorphonuclear cells scattered in the wound area and around the vessels.

In the nontreated normal mice (db/+), epidermal regeneration and remodeling of the dermis was complete and almost similar to that of diabetic treated mice. Epidermal elongation was spread over the entire wound surface. Dermal regeneration was characterized by abundant collagen bundles, similar to that of the margins of the wound, and thick elongated capillary vessels disposed vertically toward the epithelium, on the edge site of the wound (Fig 5, A). Around or in the blood vessels, there were a few inflammatory cells. Fibroblasts were disposed orderly, parallel to the wound surface, in an organized granulation tissue with dense matrix formation. Treatment with raxofelast did not significantly change the process of wound repair in healthy (db/+) mice (Fig 5, B).

Breaking strength. The wound breaking strengths for each group at day 12 are depicted in Fig 6. The breaking strength of incisional wounds from diabetic mice treated with raxofelast was higher than that of diabetic mice treated with vehicle. As a result of raxofelast administration, breaking strength in wounds from db/db mice treated with raxofelast was approximately the same as in healthy (db/+) mice. No significant differences in breaking strength were observed between healthy mice treated with raxofelast or the vehicle.

MDA measurement and MPO activity. MDA levels and MPO activity were evaluated throughout the study. Very low MDA levels, investigated as an index of lipid peroxidation, were found in db/+ mice during the wound healing process; in addition, the administration of raxofelast in these animals did not cause any modification of this parameter (Fig 7). In contrast, increased MDA levels were observed in diabetic mice treated with the vehicle, and raxofelast treatment succeeded in reducing the increased lipid peroxidation (Fig 7).

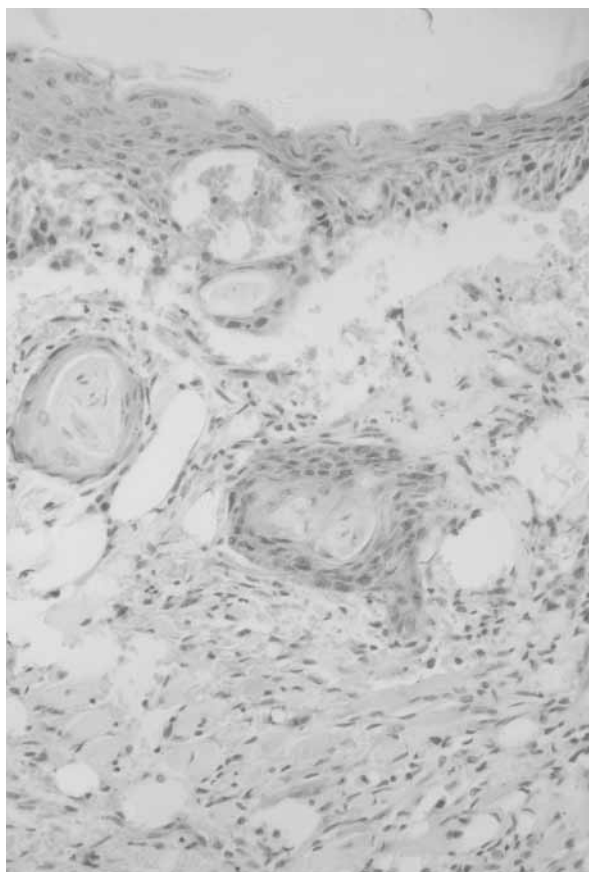


Fig 2. Histologic photomicrograph of wound specimen at day 12 from diabetic (db/db) mice treated with vehicle. Little epidermal elongation, intraepithelial bullae, and evident edema of the dermis. (Original magnification $\times 50$).

MPO activity was studied to evaluate the presence of polymorphonuclear neutrophils in the wounded skin. Low MPO activity was observed during the wound healing process in db/+ animals treated either with the vehicle or raxofelast (Fig 8). Indeed, wounded skin obtained from diabetic mice treated with the vehicle showed a marked polymorphonuclear neutrophil infiltration, and this paralleled histologic results very well. Diabetic mice treated with raxofelast showed a dramatic reduction in neutrophil infiltration in the wounded skin. This latter finding is in close agreement with the histologic report showing a blunted presence of infiltrated neutrophils in this group of rats.

Collagen content. In wounds from db/+ mice, collagen was observed to increase until day 6 after skin injury, when maximal collagen content was reached (Fig 9). At day 6, wounds from diabetic mice treated with the vehicle contained substantially less collagen than wounds from healthy

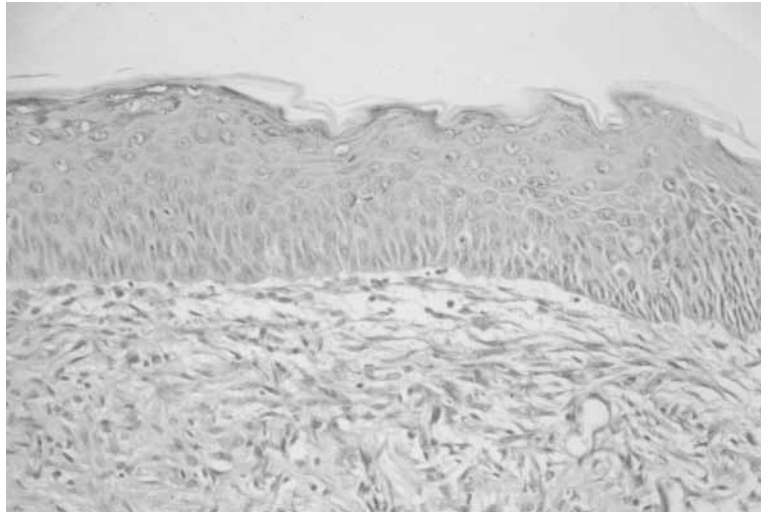


Fig 3. Histologic photomicrograph of wound specimen at day 12 from diabetic (db/db) mice treated with raxofelast. Moderate reepithelialization and well-organized granulation tissue. (Original magnification $\times 50$).

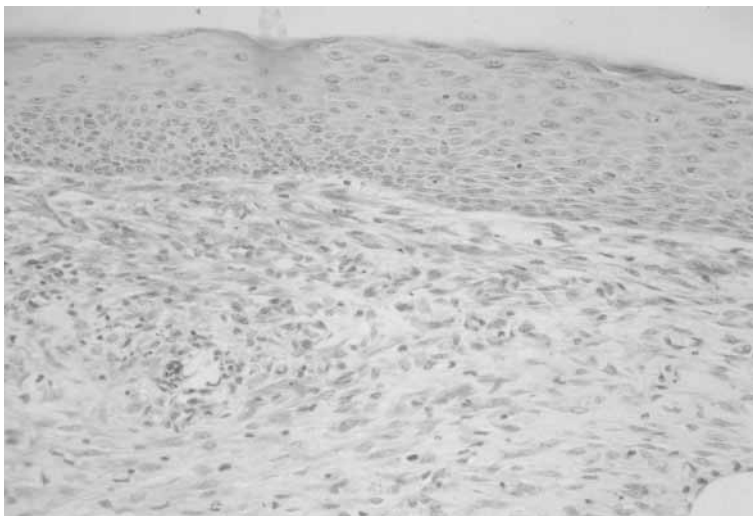


Fig 4. Histologic photomicrograph of wound specimen at day 12 from diabetic (db/db) mice treated with raxofelast. Slight edema in the superficial dermis. Fibroblasts with parallel orientation to the surface are present. (Original magnification $\times 50$).

(db/+) mice. The collagen content of wounds did not differ significantly between db/+ mice treated with raxofelast or vehicle. Raxofelast increased the collagen content of diabetic mice wounds to levels close to that observed in wounds of db/+ mice.

DISCUSSION

A decreased healing capacity in diabetes is the result of multiple factors, including elevated blood sugar levels, suppressed cell mediated immunity, local ischemia, and free radical generation. Inadequate oxygenation, such as in local ischemia, causes the production of extremely

reactive metabolites called free oxygen radicals that impair normal wound healing by damaging endothelial cells, capillary permeability, and collagen metabolism.¹⁸

Skin ischemia provides favorable conditions for the formation of oxygen-derived free radicals by means of leukocytes, mostly neutrophils, which are activated during ischemia. The release of oxygen radicals by adhered activated leukocytes causes additional damage because more neutrophils are attracted and the process is amplified.¹⁹ Under normal conditions, the generation of free radicals is counterbalanced by the presence of

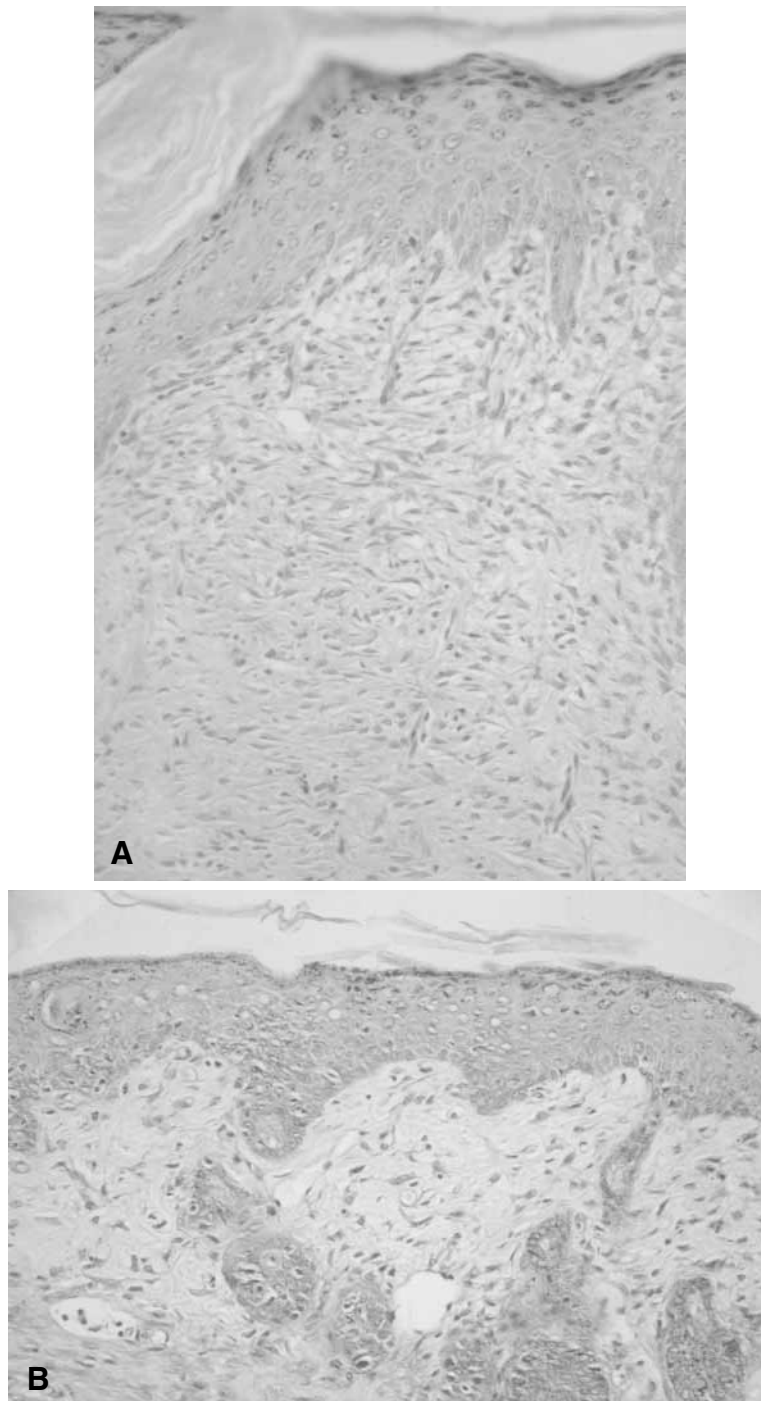


Fig 5. Histologic photomicrograph of wound specimen at day 12 from healthy (db/+) mice treated with raxofelast (A) and vehicle (B). Complete reepithelialization and well-formed granulation tissue. Vessels perpendicular oriented to epithelial surface. (Original magnification $\times 50$).

adequate endogenous antioxidant defenses,²⁰ but when the generation of free radicals exceeds the capacity of the defenses, these highly active radicals may produce structural changes that may contribute to reversible or irreversible cell injury.

Oxygen radicals cause tissue damage by lipid peroxidation of cellular and organelle membranes, disruption of the intracellular matrix, and alteration of important protein enzymatic processes.^{19,21} These agents not only damage the lipids

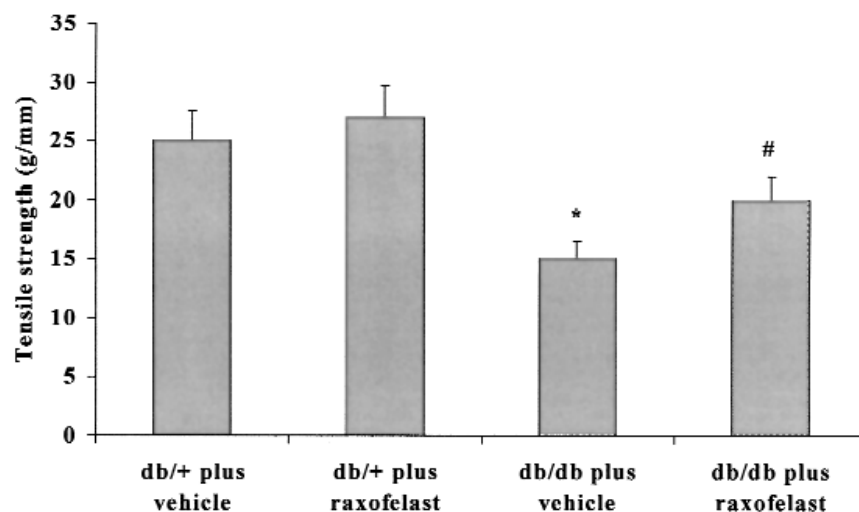


Fig 6. Tensile strength (g/mm), evaluated at day 12, in wounds obtained from db/+ and db/db mice treated either with vehicle (1 mL/kg/d, intraperitoneally) or raxofelast (15 mg/kg/d, intraperitoneally). Bar heights represent the mean \pm SEM of 7 experiments. Asterisk, $P < .01$ versus db/+ mice; number sign, $P < .01$ versus db/db mice treated with vehicle.

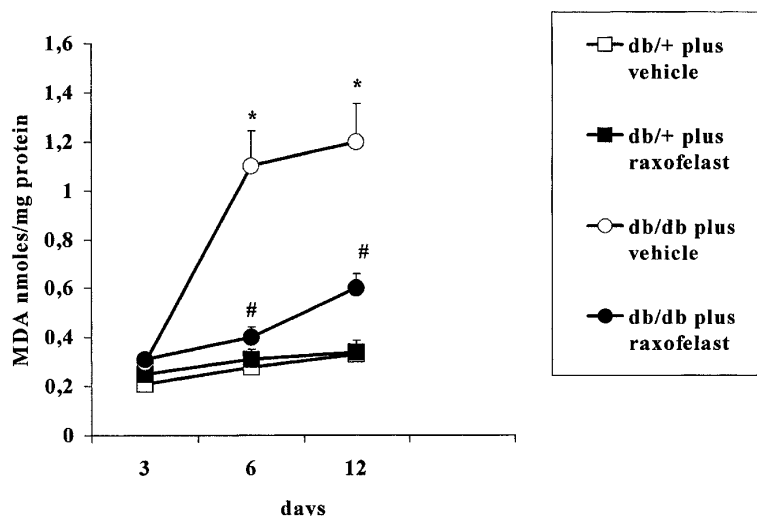


Fig 7. MDA levels in wound specimens collected at different time points from db/+ and db/db mice treated either with vehicle (1 mL/kg/d, intraperitoneally) or raxofelast (15 mg/kg/d, intraperitoneally). Each point represents the mean \pm SEM of 7 experiments. Asterisk, $P < .01$ versus db/+ mice; number sign, $P < .01$ versus db/db mice treated with vehicle.

but also produce secondary intermediates, lipid hydroperoxides, which can lead to a chain reaction of lipid peroxidation.²²

Vitamin E seems to be the most active lipid-soluble antioxidant in the human body.²³⁻²⁵ Together with the water soluble antioxidant ascorbic acid, it constitutes a regenerative system that is effective in suppressing both enzymatic and nonenzymatic peroxidation of membrane lipids by breaking the chain reaction of radical formation.²⁶⁻²⁹

The antioxidant activity of raxofelast and its deacetylated active metabolite IRFI 005 has been described in previous in vitro and vivo studies.⁶ In addition, IRFI 005 has been shown to be a scavenger of superoxide anion, with a linear dose-response curve starting from 5 μ mol/L. After systemic administration of raxofelast to rats, dogs, and humans, the plasma concentrations of the parent compound were very low, whereas high levels of IRFI 005 were found in plasma and tissue.^{30,31}

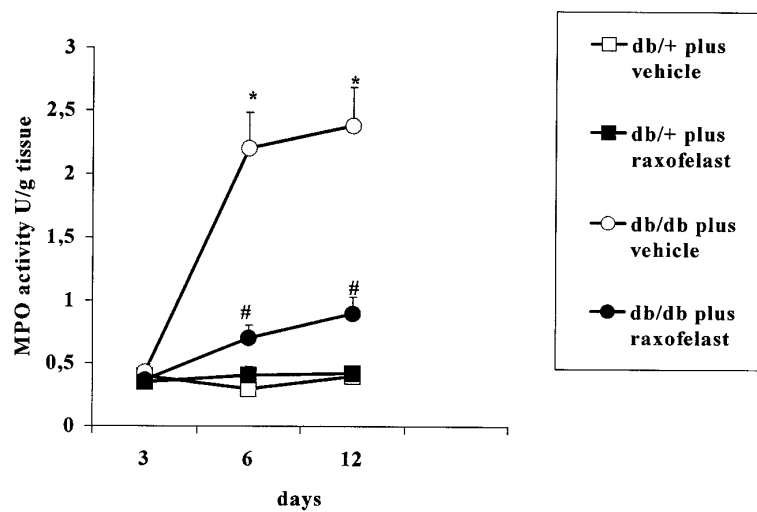


Fig 8. MPO activity in wound specimens collected at different time points from db/+ and db/db mice treated either with vehicle (1 mL/kg/d, intraperitoneally) or raxofelast (15 mg/kg/d, intraperitoneally). Each point represents the mean \pm SEM of 7 experiments. Asterisk, $P < .01$ versus db/+ mice; number sign, $P < .01$ versus db/db mice treated with vehicle.

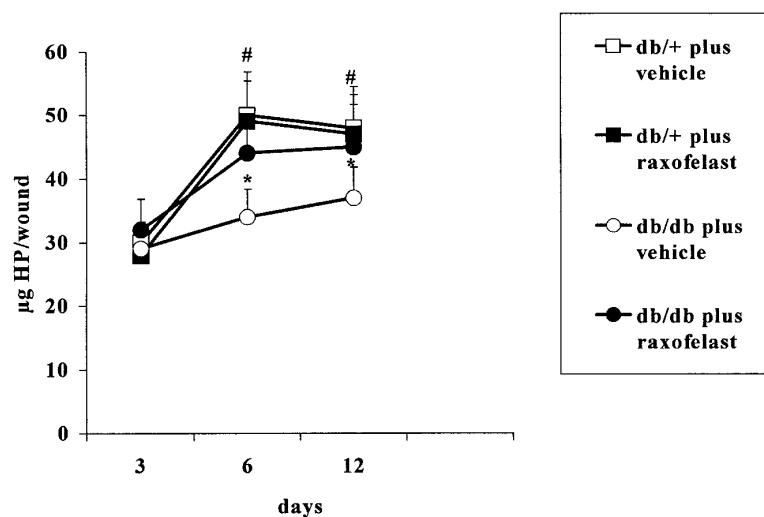


Fig 9. Hydroxyproline (HP) content in wound specimens collected at different time points from db/+ and db/db mice treated either with vehicle (1 mL/kg/d, intraperitoneally) or raxofelast (15 mg/kg/d, intraperitoneally). Each point represents the mean \pm SEM of 7 experiments. Asterisk, $P < .01$ versus db/+ mice; number sign, $P < .01$ versus db/db mice treated with vehicle.

In our model, raxofelast was able to reverse the effects of diabetes on wound healing by reducing lipid peroxidation, neutrophil infiltration, edema, and stimulating reepithelialization, neovascularization, proliferation of fibroblasts, and the synthesis and maturation of extracellular matrix. We suggest that raxofelast-induced reduction in neutrophil infiltration may be caused by lipid peroxidation inhibition, which in turn leads to a reduction in the formation of the chemotactic intermediates.

Indeed, the presence of neutrophils in the late stage of the wound healing process represents a negative event: in fact, it has been shown that persistent infiltration and activation of neutrophils may lead to an abnormal repair response having profound effects on down-stream cell migration, matrix deposition, and angiogenesis. Such an alteration (persistent neutrophil infiltration) has been shown to play a pivotal role in the delayed wound healing in aged humans.³² In agreement with this

finding, the wounds of diabetic mice had a marked MPO activity 6 and 12 days after skin injury, and this may contribute, at least in part, to the impairment of wound healing in experimental diabetes.

MDA measurement is an indicative method of evaluating lipid peroxidation.³³ The large amount of MDA found in the wound tissue of diabetic mice is consistent with the occurrence of a free-radical-mediated wound healing damage.

Lipid peroxidation is also considered responsible for the impairment of endothelial cells, capillary permeability, and fibroblast and collagen metabolism.¹⁸ Bohlen and Niggel¹⁰ reported that microvascular derangement in db/db mice is characterized by a decreased number of arterioles, loss of vascular tone, and a reduced cross-sectional area in the vessel walls. The increased permeability and the consequent marked edema in db/db mice is probably the result of the loss of endothelial cell continuity by direct action of oxygen free radicals on the lipoperoxidases of the cell membranes.

Raxofelast, protecting the polyunsaturated fatty acids in membranes against lipid peroxidation, has been shown to have angiogenic activity and to stimulate proliferation of fibroblasts and keratinocytes in db/db mice—thus the degree of wound healing approximated to that observed in the control heterozygous (db/+) mice.

In addition, the beneficial effects of raxofelast on wound healing were stressed by the increase in both breaking strength measurements and collagen content.

In conclusion, these results suggest that raxofelast is useful in improving wound healing in cases of deficient wound repair such as that which occurs in patients with diabetes.

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